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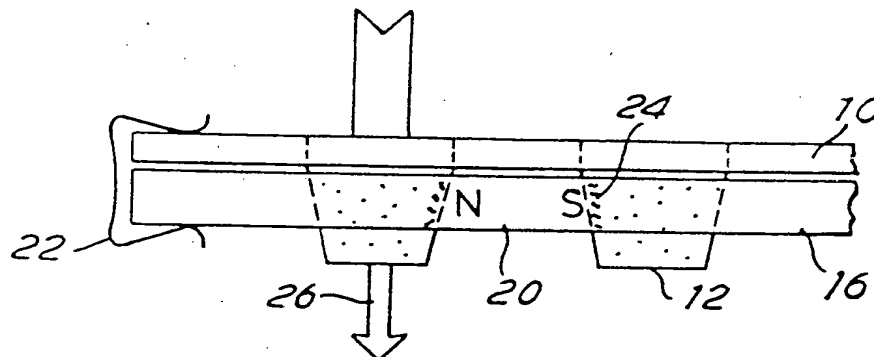
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(54) Title: TURBIDIMETRIC ASSAY



(57) Abstract

A method of performing a turbidimetric assay for an analyte which is a member of an immuno-reaction system involves the use of: first magnetically attractable particles which carry a first species reactive with a member of the immuno-reaction system, but which are not agglutinatable by the analyte; and second particles which carry a second species reactive with a member of the immuno-reaction system. The sample is incubated with the first particles and the second particles, so as to form an agglutinated product containing the first particles to an extent related to the analyte concentration. The agglutinated particles are brought down by means of a magnetic field, and the optical density of the supernatant liquid observed. When the analyte is an antigen or hapten, the first particles preferably carry a monoclonal antibody, and the second particles may carry a different monoclonal or a polyclonal antibody.

TURBIDIMETRIC ASSAY

5 This invention is concerned with agglutination
assays. Such assays are widely used to detect, or
determine the concentration of, an analyte in a
biological fluid. At least one of the assay reagents
is used in a form attached to fine solid particles
10 suspended in the liquid assay medium, which particles
are caused to agglutinate to an extent dependent upon
the concentration of the analyte in the sample. The
extent of this agglutination can be measured by two
main techniques. One involves use of a particle
counting device such as that sold by us under the Trade
15 Mark IMPACT, which is adapted to count only particles
in a specific size range. This technique has the
advantage of high sensitivity and accuracy, and is
being commercially used on an increasing scale.

The other main technique for determining the extent
20 of agglutination is a turbidimetric technique which
involves measuring the optical density of the assay
mixture. This technique has the advantages of being
cheap and quick, and can be performed on the reaction
mixture in the assay vessel. Its major disadvantage is
25 lack of sensitivity. It will be appreciated that both
the monomeric latex particles and the agglutinated
particles increase the optical density of the reaction
mixture, albeit to somewhat different extents.
Measuring optical density in order to determine
30 agglutination involves measuring this difference which
is generally rather slight. That is why the standard
method is not very sensitive.

The present invention arises from the idea that, if
the agglutinated particles could be removed from the
35 reaction mixture, the optical density of the remaining
reaction mixture would provide a much more sensitive
and accurate measure of the amount of analyte in the

sample. The invention uses magnetically attractable particles to achieve this effect.

The invention provides a method of performing a turbidimetric assay for an analyte in a sample, which analyte is a member of an immuno-reaction system, by
5 the use of:

first magnetically attractable particles which carry a first species reactive with a member of the immuno-reaction system, but which are not
10 agglutinatable by the analyte, and

second particles which carry a second species reactive with a member of the immuno-reaction system, which method comprises the steps of incubating a
-mixture of the sample with the first particles, and
15 with the second particles, together with any other required members of the immuno-reaction system, whereby agglutination takes place, to an extent related to the concentration of the analyte in the sample, resulting in the formation of an agglutinated product containing the first particles,

20 applying a magnetic field to the mixture to bring down the first particles, and
measuring the optical density of the mixture.

The nature of the analyte is not critical to the invention. It may be an antigen or an antibody.
25 Or it may be a hapten. Different conditions may need to be used, as described below, depending on whether the analyte is mono-epitopic or poly-epitopic. The nature of the sample is similarly not critical, but is frequently a body fluid such as plasma or serum.

30 Two different sets of particles are used in the method of the invention, with different reagents attached to each. The nature of the particles is not critical, and commercially available materials may be used.
35 It is necessary that the first particles be

2014

magnetically attractable and the second be not magnetically attractable. The particles may be of glass or ceramic or metal oxide, or an organic polymer e.g. polystyrene, in which case a suspension of the particles is generally known as a latex. The second particles need to be capable of changing the optical density of a fluid medium in which they are present at a concentration of, say, 1/500, for which purpose latex particles are suitable. The species carried by these particles may be attached by chemical or physical means well known in the art.

The first and second particles carry first and second species which are different from one another, but are both reactive with a member of the immuno-reaction system. When the analyte is an antigen or a hapten, the first and second species may be selected from the analyte, an antibody to the analyte, and a second antibody to the said antibody. The method involves incubating a mixture of the sample with the first particles, and with the second particles, together with any other required members of the immuno-reaction system. Depending on circumstances, it may be necessary to incubate the sample with the first and second particles in sequence, in either order, or with both sets of particles together.

There follow descriptions of six different methods of performing the mixing and incubating steps of the invention.

A. The analyte is an antigen, a poly-epitopic material, of which thyroid stimulating hormone (TSH) may be taken as an example. The first species on the first magnetically attractable particles is a monoclonal antibody to the antigen. The second species on the second particles is a polyclonal antibody to the antigen. The method involves

incubating a mixture of the sample with the first particles in excess, whereby the analyte becomes bound to the antibody on the first particles. Because this is a monoclonal antibody, agglutination does not take place at this stage.

5 Then the second particles are added, also in excess, and the system further incubated. Agglutination takes place at this stage, to an extent proportional to the amount of antigen in the sample. Because of the order of addition of the particles, all
10 agglutinated particles contain at least one of the first magnetically attractable particles.

B. This system is similar to A. The analyte is an antigen. The first magnetically attractable particles carry a monoclonal antibody to the antigen.
15 The second particles carry, not a polyclonal antibody, but another monoclonal antibody, different from that carried by the first particles, to the antigen.

The sample is mixed with the first and second particles, both in excess, and incubated. Agglutination takes place to an extent proportional to the amount of antigen in the sample. Because both
20 sets of particles carry monoclonal antibodies to the antigen, all agglutinated particles comprise both first and second particles, and therefore contain at least one of the first magnetically attractable particles.

25 C. This is a variant of the systems of A and B. As before, the analyte is an antigen and the first magnetically attractable latex carries a monoclonal antibody to the antigen. The second particles may
30 carry either a polyclonal antibody to the antigen (as in A), or a different monoclonal antibody to the antigen (as in B).

The sample is incubated with an excess of the first particles, as a result of which the antigen
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becomes bound to the particles. The mixture is spun-down, the supernatant discarded and the precipitate re-suspended in a smaller volume of liquid. The second particles are then added and the mixture incubated, as a result of which agglutination takes place as before. This variant involves reducing the volume of the reaction mixture between the first and second incubations, and is particularly attractive when the sample contains only a low concentration of analyte. It overcomes the problem that agglutination reactions are difficult to perform in very dilute solutions.

D. The analyte here is a hapten, a mono-epitopic species capable of raising antibodies only when bound to another material such as bovine serum albumin (BSA), for example a thyroid hormone such as T3 or T4. The first magnetically attractable particles carry the hapten on their surface. The second particles carry antibodies to the hapten on their surface.

The method involves incubating a mixture of a sample containing the hapten with the first and second particles. The first and second particles bind together to form agglutinated particles, and this binding is inhibited by the hapten which occupies binding sites on the antibody on the second particles. The extent of agglutination is therefore inversely proportional to the analyte concentration in the sample.

E. This is a variant on D. The analyte is a hapten and the first magnetically attractable particles carry the hapten on their surface as before. A first antibody to the hapten is used in solution. The second particles carry on their surface second antibodies (or $F(ab)_2$ fragments thereof) to the first antibody.

The method involves incubating a mixture of a

sample containing the analyte with the first antibody in solution and with the first and second particles. The F(c) portion of the first antibody becomes bound to the second particles. The hapten in the sample and the hapten bound to the first particles compete for binding with the F(ab)₂ sites of the first antibody. Agglutination involving both sets of particles takes place to an extent inversely proportional to the concentration of the analyte in the sample.

5 F. The analyte is a hapten. A first antibody to the hapten is used in solution. The second particles carry the hapten on their surface. The first magnetically attractable particles carry a monoclonal second antibody to the first antibody. Also included in the system is a latex, the particles of which carry a different monoclonal second antibody to the first antibody.

10 The method involves incubating a mixture of the sample with all the named reagents. The hapten in the sample and the hapten on the second particles compete for binding with first antibody. First antibody bound to the free hapten from the sample is available for binding by the second antibody. First antibody bound to hapten on the second particles is, by reason of steric inhibition, not able to take part in agglutination reactions involving the first particles. The two monoclonal second antibodies both bind to the first antibody, unless steric inhibition prevents this, resulting in agglutination. The extent of agglutination is directly proportional to the concentration of hapten in the sample.

20 The result of all these methods is a reaction mixture containing agglutinated particles and unagglutinated particles in relative proportions which are related in some way to the analyte concentrations in the sample. The next step is to apply a magnetic field

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to the mixture to remove the magnetically attractable first particles from suspension. Since all the agglutinated particles contain at least one of the first particles, the effect of this step is to remove from suspension all the agglutinated particles, together with any unagglutinated first particles. Unagglutinated second particles remain in suspension; the concentration of these is related in some way to the analyte concentration in the sample. The optical density of the mixture is then measured and the measurement used to detect the presence of, or determine the concentration of, the analyte in the sample. Equipment for measuring optical density is commercially available and can be used in conventional manner.

Various configurations are possible for this part of the method. For example, the assay may be performed in a cuvette with parallel optical glass side walls through which the optical density measurement is made in a horizontal direction. In this case, a magnet can be placed below the bottom of the cuvette in order to draw down the first particles; or a magnetic stirrer might be introduced into the bottom of the cuvette for the same purpose.

In another preferred embodiment, the assay may be performed in cups of a microtitre plate. In this case the optical density measurement would normally be made vertically by an instrument adapted to traverse from cup to cup of the plate. The magnetic field needs to be applied between the cups so as to pull the magnetically attractable first particles to one side of each cup.

Reference is directed to the accompanying drawings in which:

Figure 1 is a perspective diagrammatic view of

two-part equipment for effecting magnetic separation;
and

Figures 2 and 3 are diagrammatic sectional side
5 - elevations of the same equipment at different stages in
the method.

Referring to the drawings, there is shown a
microtitre plate (10) including cups (12), each of
which contains a reaction mixture (14) at the
10 conclusion of the incubating step of the invention.
Also shown is an adaptor (16) in the form of a plate
with holes (18) to receive the cups of the microtitre
plate and permanent magnets (20) positioned between the
holes.

15 As shown in figure 2, the microtitre plate (10) is
initially separate from the adaptor (16), and the
agglutinated and unagglutinated particles are
present in uniform suspension in the reaction mixture
(14).

20 In the next step as shown in figure 3, the
microtitre plate has been lowered onto the adaptor and
the two secured together with a clip (22). The magnet
has acted on the first particles in the reaction
mixture, and has drawn them, as shown at (24), to the
25 side wall of each cup. The second particles
remain in uniform suspension. The optical density of
the reaction mixture in one cup is being measured in
the direction shown by the arrow (26).

Alternatively, having used a magnet to draw down
30 the first particles, it is possible to transfer the
supernatant liquid to some other vessel for measurement
of optical density. This may provide increased
accuracy and precision, albeit at added expense.

A system where the optical density of the mixture
35 is measured only after the magnetically attractable
particles have been completely brought down, may
be regarded as an equilibrium one. It may alternatively

be possible to perform the optical density measurement kinetically, by observing the rate or extent of the change of optical density of the mixture when the magnetic field is applied to it.

The following examples illustrate the invention. Example 1 is included by way of comparison.

Example 1

For this experiment there were used serum samples containing known concentrations of TSH. $F(ab)_2$ antibody fragments were coupled via a chemical spacer to 0.2 micron latex. 0.03ml of the sample was mixed with 0.03ml of a 0.01% suspension of the latex particles in buffer and the mixture incubated, as a result of which agglutination took place to an extent determined by the concentration of TSH in the sample. The optical densities of various reaction mixtures were measured, and were found to vary by about 10% over the usual range of concentrations (up to 50ng/ml) expected for TSH. Using these figures it was possible to construct a dose-response curve, but one which was rather flat and therefore insensitive to differences in TSH concentration in unknowns.

By passing a sample of the reaction mixture through a particle counter set to count unagglutinated particles, results of greatly improved precision and sensitivity were obtained.

Example 2

The samples were again of serum containing various known concentrations of TSH. The first magnetically attractable latex particles carried a monoclonal antibody to the TSH. The second latex particles carried a polyclonal antibody to TSH.

0.05ml of sample were digested with 0.1ml pepsin in 0.1ml water for 4 minutes. Then 1ml glycine buffered saline (GBS)/BSA 1% was added, together with 0.05ml of tris buffer and 0.03ml of a 0.1% suspension of the

first latex. The mixture was incubated to cause TSH from the sample to bind to antibody on the magnetic latex.

5 To 0.08ml of the resulting mixture was added 0.03ml of a 0.065% suspension of the second latex in buffer. This mixture was incubated for one hour, resulting in agglutination of particles to an extent proportional to the TSH concentration in the sample. Then a magnetic
10 field was applied to bring down the magnetic latex in the mixture. The optical density of the supernatant liquid was measured, the results obtained using various standards were plotted on a graph to give a dose-response curve of excellent slope for a sensitive and
15 accurate assay.

The experiment was repeated, but using 0.5ml of sample in place of 0.05ml. After the first incubation with the magnetic latex; the mixture was spun down and the bulk of the supernatant liquid removed. This
20 resulted in an assay with increased sensitivity.

Example 3

As before, serum samples were used containing known concentrations of TSH. The assay was performed using the magnetic latex carrying monoclonal antibody as in
25 example 2, and a 0.8 micron latex coated with a second monoclonal antibody to TSH (but from another clone). This permitted the assay to be performed using a single incubation.

There were mixed together: 0.03ml of the sample in
30 buffer; 0.03ml of a suspension of the magnetic latex; and 0.03ml of the 0.8 micron second latex. The mixture was incubated for one hour, as a result of which agglutination took place to an extent proportional to the TSH concentration in the sample. A magnetic field
35 was applied to the mixture for one minute. 1ml of buffer was added and the optical density of the supernatant mixture measured. The results obtained

enabled an excellent dose-response curve to be constructed.

5 This experiment also demonstrates that an assay for TSH can be performed without the need for initial pepsin digestion of the sample.

Reference is directed to figure 4 of the accompanying drawings, which is a graph of optical density, expressed in arbitrary figures, against TSH
10 concentration in the sample, measured as ng/ml. The graph includes three dose-response curves which were obtained as follows:

Curve A was obtained by a method as described in comparative Example 1 above and without use of a
15 magnetic separation technique. The curve is substantially horizontal for much of its length, indicating that the assay is insensitive.

Curve B was obtained by an experiment according to Example 2 above. Note that the dose-response curve has
20 a useful slope at TSH concentrations from 0 up to 100 ng/ml.

Curve C was obtained by an assay similar to that used for Curve B, but the measurements were made using an IMPACT particle counter. Below 5 ng/ml TSH the
25 slope is rather small; from 5 to 100 ng/ml the sensitivity is comparable to that achieved by Curve B.

The method of this invention, involving an agglutination assay and measurement of optical density, has the following advantages:

30 a) It eliminates the step of sample transfer from one tube to another. Sample transfer involves expense and time, and can damage agglutinated particles which are not robust. Also sample transfer always raises carry-over problems from one tube to the next.

35 b) It is very quick. Measurements can be made rapidly and vertically by moving the instrument head from one microtitre well to the next.

c) It is at least as sensitive as a particle counting assay.

5 d) It is enormously faster than immuno assays which depend on radioactive or fluorescent or luminous labels.

e) It is more sensitive than RIA and comparable in this respect to fluorometric or luminescent immuno assays.

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CLAIMS

1. A method of performing a turbidimetric assay for an analyte in a sample, which analyte is a member of an immuno-reaction system, by the use of:
- 5 first magnetically attractable particles, which carry a first species reactive with a member of the immuno-reaction system, but which are not agglutinatable by the analyte, and
- 10 second particles which carry a second species reactive with a member of the immuno-reaction system, which method comprises the steps of incubating a mixture of the sample with the first particles, and with the second particles, together with any other
- 15 required members of the immuno-reaction system, whereby agglutination takes place, to an extent related to the concentration of the analyte in the sample, resulting in the formation of agglutinated particles containing the first particles,
- 20 applying a magnetic field to the mixture to bring down the first particles, and measuring the optical density of the mixture.
2. A method as claimed in Claim 1, wherein the analyte is an antigen.
- 25 3. A method as claimed in Claim 1, wherein the analyte is a hapten.
4. A method as claimed in Claim 2, wherein the analyte is thyroid stimulating hormone.
5. A method as claimed in Claim 2 or Claim 4, wherein
- 30 the particles of the first magnetically attractable particles carry a monoclonal antibody to the analyte.
6. A method as claimed in Claim 5, wherein the second particles carry a different monoclonal antibody to the analyte.

7. A method as claimed in Claim 6, wherein the sample is mixed with suspensions of the first and second particles and the mixture incubated.

5 8. A method as claimed in Claim 5, wherein the second particles carry a polyclonal antibody to the analyte.

9. A method as claimed in Claim 6 or Claim 8, wherein the sample is first incubated with the first particles, then concentrated, and then incubated with the second particles.

10 10. A method as claimed in any one of Claims 1 to 9, wherein the reaction mixture is contained in wells of a microtitre plate, with optical density measurements being made vertically through successive wells.

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INTERNATIONAL SEARCH REPORT

International Application No PCT/GB88/00614

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁴ : G 01 N 33/553, 33/543, 33/536, 33/546		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC ⁴	G 01 N	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT *		
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X Y	EP, A1, 0 194 156 (SANKO JUNYAKU CO., LTD., TMM) 10 September 1986 whole document see in particular p. 4, line 22, p. 5, lines 21-32 --	1, 9 2-8, 10
Y	US, A, 4 115 535 (I. GIAEVER) 19 September 1978 whole document --	1-4, 7, 8
Y	EP, A1, 0 201 755 (ALLIED CORPORATION) 20 November 1986 whole document see in particular examples and claims 1, 4, 8 and 9 --	1-8
X Y	US, A, 4 279 817 (P. L. MASSON et al.) 21 July 1981 whole document see in particular column 3, lines 21-43, column 4, lines 24-30 and claims --	1, 2 3-10
.../...		
<p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
12 October 1988		01 DEC 1988
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		 P.C.G. VAN DER PUTTEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	GB, A, 1 590 525 (TECHNICON INSTRUMENTS CORPORATION) 3 June 1981 whole document see in particular page 3, lines 14-21 and 55 ---	1-3
A	Patent Abstract of Japan, Vol. 10, No. 322 P511, abstract of JP 61-128168, publ. 1986-06-16 *abstract* ---	1
A	WD, A1, 86/04684 (LAB-SYSTEMS OY) 14 August 1986 whole document ---	1
A	DE, A1, 33 23 137 (ABBOTT LABORATORIES) 5 January 1984 whole document ---	1-9

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

PCT/GB88/00614
SA23492

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 01/09/88. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0194156	10-09-86	JP-A- 62070764	01-04-87
US-A- 4115535	19-09-78	None	
EP-A- 0201755	20-11-86	JP-A- 61265571	25-11-86
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US-A- 4279617	21-07-81	FR-A- 2449892	19-09-80
		GB-A- 2045431	29-10-80
		DE-A- 3006899	11-09-80
		JP-A- 55156866	06-12-80
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		JP-A- 53104726	12-09-78
		CA-A- 1089360	11-11-80
JP-A- 61128168	16-06-86	None	
WO-A- 86/04684	14-08-86	None	
DE-A- 3323137	05-01-84	None	

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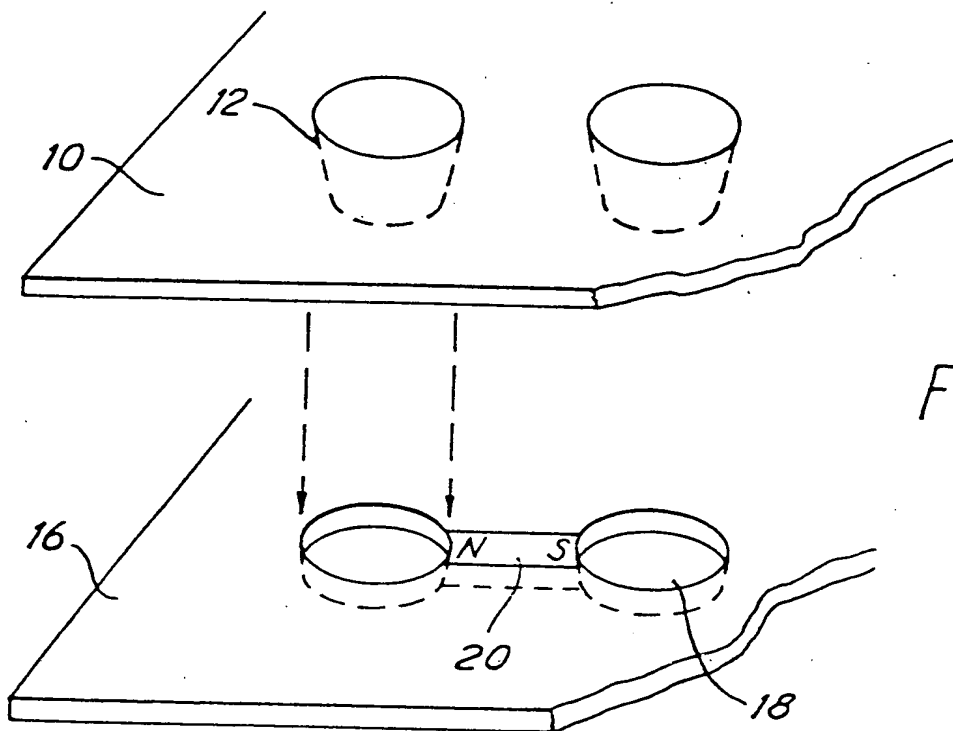


FIG. 1

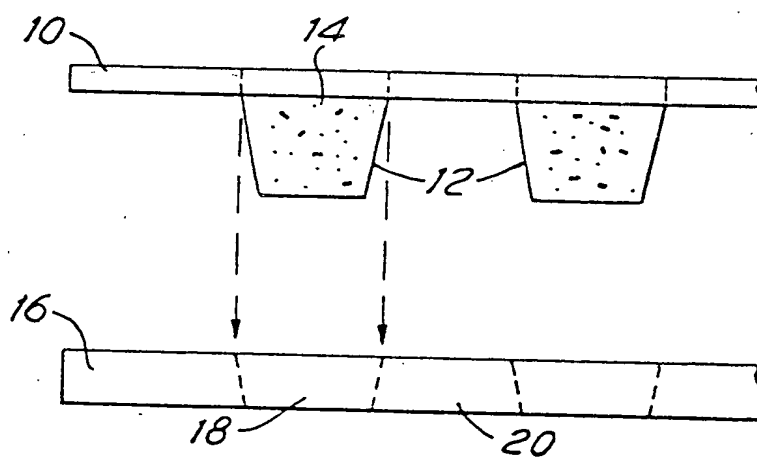


FIG. 2

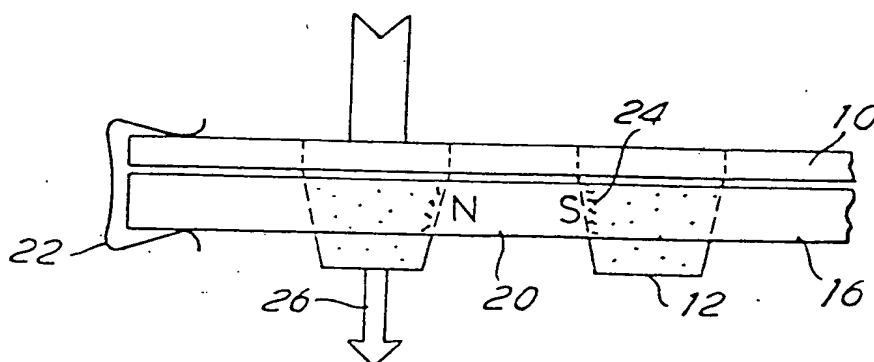


FIG. 3

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FIG. 4

